Discussion

Detecting the *MYCN* status of NB is the standard of care in the diagnosis and treatment of the disease. In the Study Group of Japan for Advanced NB (JANB), two chemotherapeutic regimens for advanced NB have been designed based on the *MYCN* amplification status (more than 10 copies of *MYCN* or less than 10 copies of *MYCN*) since 1991 [13]. Recently, FISH and Q-PCR have recommended determining *MYCN* amplification status instead of SB. In this study, a small number of *MYCN* amplified cells and MYCN gain cells detected by FISH and LCM could not be detected by SB.

However, for determining the *MYCN* status, the FISH method and Q-PCR method are controversial. With FISH, it is possible to determine the *MYCN* gene status of the individual cells, but it is only possible to evaluate small number of cells in the tumor sample. Therefore, it is difficult to determine the copy number of the *MYCN* gene throughout the tumor. Furthermore, in one study, the *MYCN* status determined by FISH was not identical to the *MYCN* status determined by Q-PCR because the background contaminated debris showed *MYCN* probe signals [14]. In addition, the tumor cells with one copy of the *MYCN* gene cannot be distinguished from peripheral blood leukocytes and normal stromal cells by FISH.

Case 10 with a large number of *MYCN* gain cells detected by FISH showed a slight increase of gene dosage of *MYCN* in a frozen block sample and all LCM areas with Q-PCR. *MYCN* gain is defined as a less than the 4-fold increase of *MYCN* signals in relation to the number of chromosomes 2 centromere signals in FISH [12]. The outcome of the patients with *MYCN* gain remains unclear. In a previous report, the 3-year event free survival of the patients with *MYCN* gain was poor in comparison to the NB patients without *MYCN* amplification, but no significant difference in the 3-year overall survival was observed [15].

In Q-PCR, the *MYCN* gene status of highly amplified *MYCN* in NB is detected accurately in comparison to other methods. On other hand, a low level of amplified *MYCN* status was unclear. In this study, 12 of 54 NB cases with a single copy of *MYCN* based on SB showed a slight increase in the gene dosage of *MYCN* by the Q-PCR. Of these 12 cases, FISH or Q-PCR in LCM areas explained the slight increase of *MYCN* in only 4 cases. In eight other cases, no *MYCN* amplified cells or *MYCN* gain cells by FISH, nor any small areas with an increase of the *MYCN* gene by LCM could be detected. And, in cases 2 and 12, the tumors with *MYCN* amplification by FISH did not show an increase in the *MYCN* gene dosage detected by LCM. This may have been due to by the intratumor heterogeneity in NB. The paraffin-embedded samples were examined by LCM, the frozen samples were examined by Q-PCR, and fresh samples were examined by FISH. We think that a small number of the *MYCN* amplified cells might thus have been contained in the frozen and block samples, however, no such cells might be observed in the paraffin-embedded samples. In previous reports, 12–27% NB patients showed heterogeneous tumors using FISH and Chromogenic in situ Hybridization (CISH) [16, 17]. This result is consistent with the present study (12/63; 19%).

Case 1, with a single copy of *MYCN* based on SB has an intratumoral necrosis. This necrotic area did not show an increase of the gene dosage of *MYCN*. It is important to analyze variable tumor cells to detect *MYCN* gene and other genetic markers. However, it is difficult to distinguish variable tissue by only macroscopic investigation in some cases. Microscopic analysis by LCM is a highly sensitive method for assessing the status of the MYCN gene in NB. However, an analysis using LCM is not practical in a routine investigation for *MYCN* amplification. A diagnostic analysis based on a combination of the Q-PCR for a block sample and dual color FISH is recommended as the standard method for assessing the status of the *MYCN* gene in NB.

A slight increase for the gene dosage of *MYCN* by Q-PCR may indicate that NB tissue contains a small number of cells with the *MYCN* amplification or a large number of cells with the *MYCN* gain, which are associated with the aggressive progression of NB. If there is a slight increase in the *MYCN* gene in the block sample indicated by Q-PCR and no *MYCN* amplified cells are detected by FISH, we recommend that an LCM investigation should be conducted to determine the *MYCN* status of variable tumor cells accurately.

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ORIGINAL ARTICLE

Complete elimination of established neuroblastoma by synergistic action of γ-irradiation and DCs treated with rSeV expressing interferon-β gene

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Dendritic cell (DC)-based immunotherapy has been investigated as a new therapeutic approach to intractable neuroblastomas; however, only limited clinical effect has been reported. To overcome the relatively low sensitivity of neuroblastomas against immunotherapy, we undertook a preclinical efficacy study to examine murine models to assess the combined effects of γ-irradiation pretreatment and recombinant Sendai virus (ts-rSeV/dF)-mediated murine interferon-β (mIFN-β) gene transfer to DCs using established c1300 neuroblastomas. Similar to intractable neuroblastomas in the clinic, established c1300 tumors were highly resistant to monotherapy with either γ-irradiation or DCs activated by ts-rSeV/dF without transgene (ts-rSeV/dF-null) that has been shown to be effective against other murine tumors, including B16F10 melanoma. In contrast, immunotherapy using DCs expressing mIFN-β through ts-rSeV/dF (ts-rSeV/dF-mIFNβ-DCs) effectively reduced tumor size, and its combination with γ-irradiation pretreatment dramatically enhanced its antitumor effect, resulting frequently in the complete elimination of established c1300 tumors 7–9 mm in diameter, in a high survival rate among mice, and in the development of protective immunity in the mice against rechallenge by the tumor cells. These results indicate that the combination of ts-rSeV/dF-mIFNβ-DCs with γ-irradiation is a hopeful strategy for the treatment of intractable neuroblastomas, warranting further investigation in the clinical setting.
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Keywords: *dendritic cell; recombinant Sendai virus; irradiation; neuroblastoma*

Introduction

Neuroblastoma, with its many clinical and molecular faces, is the most common extracranial malignant solid tumor seen in children.[1] When occurring in infants less than 1 year old, neuroblastoma shows a relatively good prognosis, whereas only 30% of children above that age with advanced cases of the disease do not experience disease progression for at least 3 years after treatment.[2] Recent efforts by physicians have demonstrated that surgical intervention, irradiation and intensive chemotherapy followed by stem cell transplantation improved the survival of such patients.[3,4] However, in a large number of these children, especially in cases with *MYCN* amplification, the disease remains intractable.[3,4]

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As an alternative potential therapy, clinical evaluation of dendritic cell (DC)-based immunotherapy was initiated several years ago. The first reported clinical study, which enrolled 15 children with advanced solid tumors including three individuals with neuroblastoma, demonstrated modest antitumor responses.[5] Subsequently, another group reported the results of tumor RNA-loaded DC vaccination for 11 patients with stage 4 neuroblastoma.[6] Even though these challenging clinical studies showed specific antitumor immune reactions, the clinical outcome is still far from the level required for a standard therapy.

Very importantly, these frontier studies suggested that the immunosuppressed condition of these patients after intensive chemotherapy might limit the efficacy of DC-based immunotherapy.[6] In addition, neuroblastoma is shown to be less immunogenic,[7] in association with a suppressed expression of major histocompatibility complex class I, which can be caused by *MYCN* amplification.[8–10] DC-based cancer immunotherapy, moreover, is now also a developing technology and has shown limited clinical outcome in other malignancies. Therefore, scientists and physicians should elucidate (1) the

most effective DC subtypes, (2) the optimal conditions and activation stimuli to generate activated DCs showing optimal antitumor effects *in vivo*, (3) the optimal route for administration and (4) the optimal dose and frequency of DC vaccinations.[11–13] The lack of such information may explain the limited efficacy of DC-based immunotherapy for advanced neuroblastoma in the clinical setting.

Recently, we have demonstrated a dramatic improvement in the efficacies of DCs activated by recombinant Sendai virus (rSeV), namely 'immunostimulatory virotherapy', on multiple syngeneic mouse models bearing highly malignant tumors, including B16F10 melanoma,[14] MH134 hepatocellular carcinoma[14] and SCCVII squamous cell carcinoma.[15] rSeV is a novel and powerful gene transfer modality as a cytoplasmic gene expression system[16–18] that leads DCs to highly activated/mature state through a DExD/H-box RNA helicase, retinoic acid-inducible gene-I (RIG-I).[19,20] Therefore, we hypothesized that rSeV-activated DCs might enhance antitumor immunity against less immunogenic c1300 neuroblastoma.

With this background in mind, we here examined and optimized the antitumor effect of DC immunotherapy activated by a 'temperature-sensitive mutant' and F-gene-deleted non-transmissible rSeV (ts-rSeV/dF), an advanced vector design showing a less cytopathic effect,[18,21,22] that is now available for mass production according to the good manufacturing practice guidelines.

Our goal was to develop therapeutics based on ts-rSeV/dF-DCs that could completely eliminate, rather than merely shrink, established tumors as well as induce protective antitumor immunity against recurrence. Importantly, although here we confirmed that the c1300 tumor was still highly resistant to DC-based immunotherapy, even with the use of DCs activated by ts-rSeV/dF, we here found that γ-irradiation pretreatment accompanying murine interferon-β (mIFN-β) gene transfer dramatically and synergistically enhanced the antitumor immunity induced by intratumoral (i.t.) injection of ts-rSeV/dF-DCs without any antigen loading *ex vivo*. We here show that this new regimen resulted not only in the complete elimination of a high proportion of established c1300 tumors 7–9 mm in diameter, but also in the induction of tumor-specific protective immunity.

Results

c1300 neuroblastoma is highly resistant to DC-based immunotherapy

Throughout this study, DCs were not pulsed by tumor antigen *ex vivo*, because antigen loading did not enhance the antitumor effect of ts-rSeV/dF-DCs that were administered i.t. to c1300 tumors (data not shown). These findings were similar to those of our earlier studies using B16 melanoma[14] and SCCVII squamous cell carcinoma.[15]

To optimize the dose of DC-based immunotherapy that was activated by ts-rSeV/dF-null (ts-rSeV/dF-DCs), we first determined the effective dose of DCs to dermally implanted c1300 neuroblastomas in the abdominal wall of A/J female mice. We here administered ts-rSeV/dF-DCs without antigen pulsation i.t., because this injection route showed an optimal antitumor effect against both B16F10 melanoma[14] and SCCIIV squamous cell carcinoma[15] in our earlier studies. At the same time, the therapeutic effect against c1300 was directly compared with that against B16F10 melanoma.

As shown in Figure 1, in the 'early treatment regimen',[14] three-times weekly administration of ts-rSeV/dF-DCs, the highest efficacy against B16F10 melanoma occurred at 10^6 cells per dose of ts-rSeV/dF-DCs, resulting in the elimination of 50% of the tumors. In contrast, ts-rSeV/dF-DCs showed a modest suppression of tumor growth of c1300 neuroblastomas without eliminating any of the tumors, suggesting that ts-rSeV/dF-DC-based immunotherapy was more effective against B16F10 than against c1300.

Gene transfer of mIFN-β by ts-rSeV/dF to DCs enhances the antitumor effect against c1300

To overcome the limited efficacy of ts-rSeV/dF-DCs against c1300, we next examined the effect of mIFN-β gene transfer by ts-rSeV/dF to DCs (ts-rSeV/dF-mIFNβ-DCs), which has been shown to enhance antitumor immunity to B16 melanoma effectively.[14]

As shown in Figure 2a, recombinant mIFN-β or human IFN-β was effective in upregulating major histocompatibility complex class I antigen expression not only in murine (c1300) but also in human (SK-N-SH and IMR32) neuroblastoma cell lines, irrespective of predefined *N-myc* amplification. However, direct cytoxicity had no or only a modest effect on them (Figure 2b). These findings were similar to those of our earlier study obtained by the use of melanomas[14] and indicated the modest susceptibility of c1300 neuroblastomas to mIFN-β.

In turn, the use of ts-rSeV/dF-mIFNβ, instead of the ts-rSeV/dF-null vector, as a DC activator dramatically enhanced the antitumor effect on c1300, resulting in the elimination of 60% of the tumors in the 'early' treatment regimen *in vivo*, as expected (Figure 3a, left three panels and Figure 3b, left graph).

We then asked whether or not established c1300 tumors, 7–9 mm in diameter, could respond to ts-rSeV/dF-mIFNβ-DCs through the 'later' treatment regimen starting at 10 days after tumor inoculation. Although ts-rSeV/dF-mIFNβ-DCs significantly suppressed the growth of established c1300 tumors, the percentage of tumors eliminated was still and unexpectedly low (one of six animals showed complete elimination; Figure 3a, right three panels and Figure 3b, right graph). In this experiment, no animal except the one showing tumor elimination survived over 120 days (data not shown; the representative data are shown in Figure 5b), indicating that rSeV/dF-mIFNβ-DCs contributed to the significant suppression of tumor growth, though not enough to prolong the survival of tumor-bearing mice.

Synergistic sensitization of c1300 tumor to ts-rSeV/dF-IFNβ-DCs by γ-irradiation pretreatment

Together, these data confirmed the limited efficacy of ts-rSeV/dF-DC-based immunotherapy against less-immunogenic c1300 neuroblastoma. We therefore next looked for a possible sensitizer that might enhance the effect of DC immunotherapy. Recent studies have demonstrated that radiotherapy induces an 'abscopal effect' against distant tumors,[23,24] probably due to the enhancement of antitumor immunity.[25] We therefore examined the combined effect of a clinically available dose of γ-irradiation (4 Gy day^{-1} for 3 days) followed by weekly

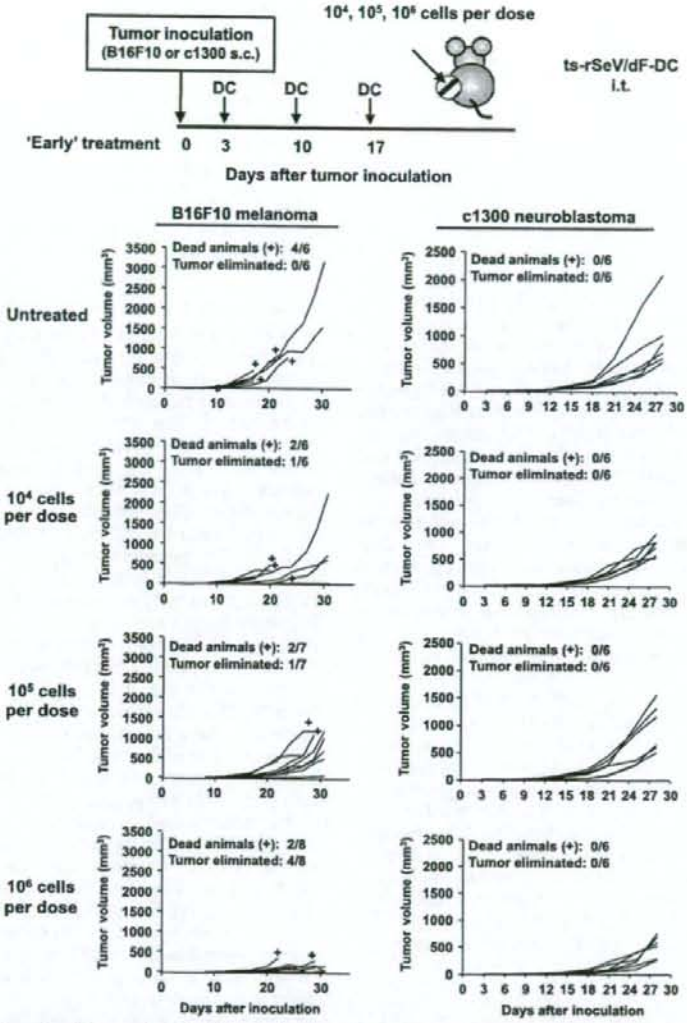


Figure 1 Direct comparison of antitumor effect by direct and repeated i.t. injection of DCs activated by temperature-sensitive mutant and F-gene-deleted non-transmissible recombinant Sendai virus (ts-rSeV/dF-DCs) without any therapeutic gene. Three days after intradermal inoculation of B16F10 melanoma (left panels) or c1300 neuroblastoma (right panels), various amounts of ts-rSeV/dF-DCs were injected weekly through an i.t. route as the 'early' treatment regimen. Thereafter, the tumor volume was measured. Lines on the panels indicate time courses of tumor volume in individual animals. Apparent dose-efficacy response was seen in the tumor volume of B16F10 melanoma, and four of eight animals that received 10^6 DCs per dose showed complete tumor elimination (left panels). In contrast, no animals inoculated with c1300 neuroblastoma demonstrated either complete elimination or significant dose–response on tumor size at any dose. The + symbol indicates animals that died during observation. DC, dendritic cell; i.t., intratumoral; rSeV, recombinant Sendai virus.

i.t. administration of rSeV/dF-mIFNβ-DCs in the 'later' treatment regimen (Figure 4, scheme). In this experiment, the tumor was intradermally implanted in the thigh to avoid radiation-induced toxicity to vital organs.

As shown in Figures 4a and b, monotherapy consisting of either γ-irradiation or rSeV/dF-mIFNβ-DCs effectively reduced the tumor volume. However, the established tumors were rarely eliminated (γ-irradiation: 0/6 animals; rSeV/dF-mIFNβ-DCs: 2/8 animals). In contrast, when these therapies were combined, six of eight animals showed the complete elimination of all established tumors at 38 days after inoculation. As the result, 5 of the 8 animals treated with both γ-irradiation and rSeV/dF-mIFNβ-DCs survived over 200 days in tumor-free condition ($P<0.001$ vs other groups) (Figure 4c).

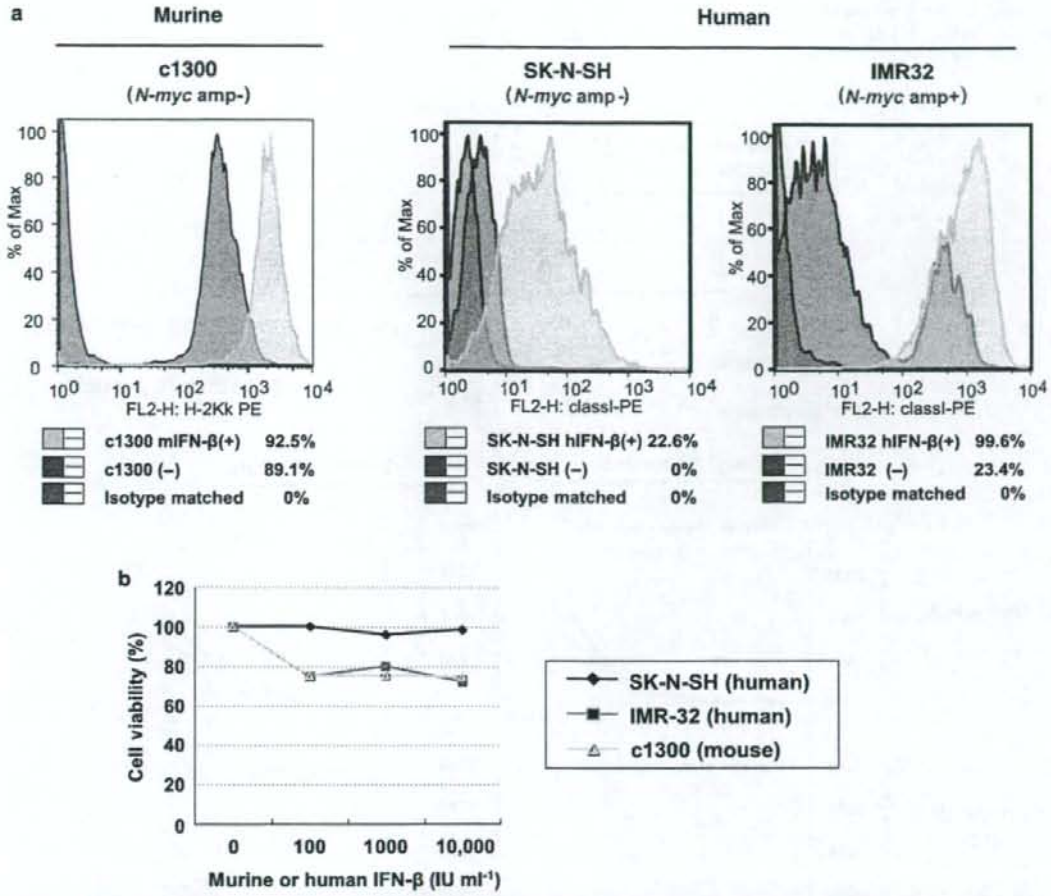


Figure 2 Interferon-β strongly upregulates MHC class I molecules of, and shows no or modest cytotoxicity against, murine and human neuroblastomas. c1300 (mouse), SK-N-SH (human) or IMR32 (human) neuroblastoma was treated with IFN-β at 1000 U ml^{-1} for corresponding species (c1300 for mIFN-β and SK-N-SH or IMR32 for hIFN-β, respectively) for 48 h. (**a**) Expression of MHC class I molecule. The panels are the typical FACS patterns among three independent experiments. C1300 cells abundantly expressed MHC class I, and mIFN-β upregulated mean fluorescence intensity (MFI). SK-N-SH cells show no expression of MHC class I, and hIFN-β stimulated its expression. IMR32 indicated dual peaks of MHC class I expression, suggesting that this cell line was composed of heterogeneous populations, and hIFN-β treatment strongly induced MHC class I expression. (**b**) *In vitro* cytotoxicity assay for IFN-βs to murine neuroblastoma (yellow line) and human neuroblastomas (SK-N-SH: blue line and IMR32: red line). The viability of SK-N-SH cells was not affected by hIFN-β, and c1300 and IMR32 showed modest (~20%) cytotoxicity by IFN-β treatment. hIFN-β, human IFN-β; MHC, major histocompatibility complex; mIFN-β, murine interferon-β. (See online version for color figure.)

Immunotherapy using ts-rSeV/dF-IFNβ-DCs was required to establish protective immunity for the second challenge of c1300 tumor cells

Finally, we asked whether or not the complete elimination of c1300 tumors might contribute to the establishment of long-lasting protective immunity.

At first, we assessed the cytotoxic T-lymphocyte (CTL) activity using splenocytes from mice with DC treatment. A strong and c1300-specific cell lytic activity of stimulated splenocytes with tumor antigen was found only in the case of mice with combined treatment, but not with other treatment groups (Figure 5a, left graph). Such a cell lytic activity could not be found when MuSS (a third-party tumor: A/J mouse-derived malignant fibrous histiocytoma)[26] was used as the target (Figure 5a, right graph).

Next, we performed an additional experiment for the second challenge by the simultaneous inoculation of c1300 and MuSS on day 186. The overdose irradiation group (34 Gy × 3 days) that showed a high percentage of c1300 tumor elimination (>70%, according to our repeated preliminary study) was also included as a control group.

As shown in Figure 5b, none of the animals bearing established c1300 tumors without any treatment or with a clinically available dose of radiation (4 Gy × 3 days) survived over 120 days after tumor inoculation. Only one of four animals treated with rSeV/dF-mIFNβ-DCs

showed tumor-free survival over 120 days, but this mouse accepted not only third-party MuSS but also c1300 at the second challenge. Three of the four tumor-bearing mice that were treated with overdose irradiation survived over 120 days in tumor-free condition, but no mouse could reject both MuSS and c1300, indicating that

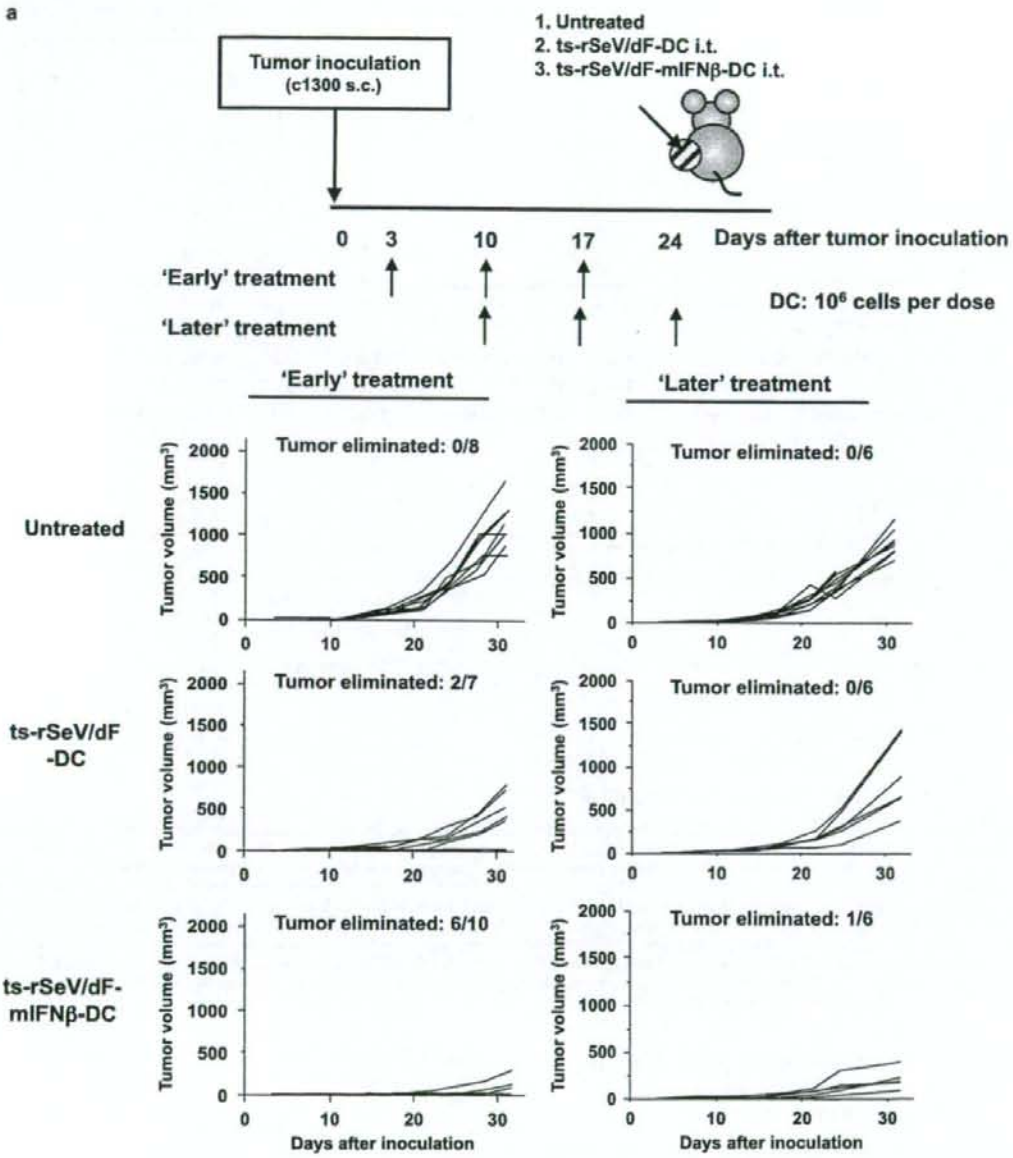


Figure 3 Enhanced antitumor activity against established c1300 neuroblastoma *in vivo* by ts-rSeV/dF-DCs expressing mIFN-β. Three days ('early' treatment regimen) or 10 days ('later' treatment regimen) after intradermal inoculation of c1300 neuroblastomas, 10^6 cells of ts-rSeV/dF-DCs with or without exogenous mIFN-β expression were injected weekly through the i.t. route. Thereafter, the tumor volume was measured. Note that all tumors treated in the 'later' regimen were over 7 mm in diameter at day 10. (**a**) Time courses of tumor volume in individual animals by 'early' (left panels) and 'later' (right panels) treatment regimens. The c1300 tumors were still resistant to the weekly treatment with ts-rSeV/dF-DCs in both regimens (middle two panels), and apparent efficacies with regard to volume reduction and tumor elimination were found in the use of ts-rSeV/dF-DC-associated exogenous mIFN-β. (**b**) Panels showing c1300 tumor volume on day 31 in the animals demonstrated in (**a**). Note that animals showing a complete elimination of tumors were excluded from these analyses. Treatment with ts-rSeV/dF-DCs significantly inhibited tumor growth, and the expression of exogenous mIFN-β strongly reduced the tumor size in both regimens. *$P<0.001$. DC, dendritic cell; mIFN-β, murine interferon-β; rSeV, recombinant Sendai virus.

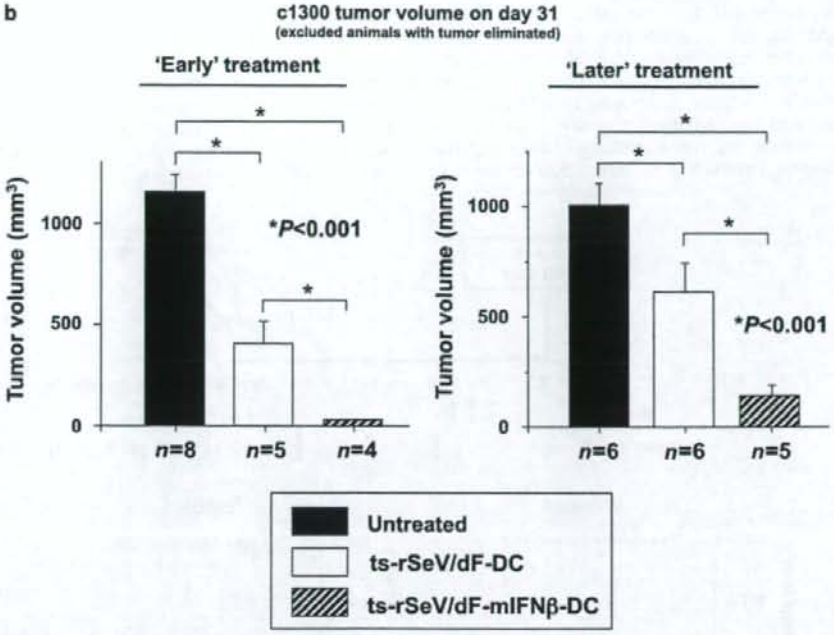


Figure 3 Continued.

tumor elimination by γ-irradiation itself does not significantly contribute to the establishment of protective immunity. In contrast, combination therapy of radiation and rSeV/dF-mIFNβ-DCs also resulted in tumor elimination and tumor-free survival over 120 days in three of the four animals, and all the four mice demonstrated complete and tumor-specific rejection to c1300 tumor inoculation, but none of them showed such a rejection of third-party, MuSS cells.

These results indicate that the synergism of combining radiation and rSeV/dF-mIFNβ-DCs contributes greatly not only to tumor reduction but also to the establishment of long-lasting and protective antitumor immunity.

Discussion

Since the early reports describing the efficacy of DC-based immunotherapy on subjects with malignancies,[27,28] this therapeutic mode has been evaluated all over the world. Recurrent neuroblastoma, which is highly resistant to currently available surgery, chemotherapy and radiotherapy, has also been a target of DC-based immunotherapy; but recent clinical studies have failed to show significant improvements in outcome.[5,6] To overcome the current limitation of this mode, we recently developed a new concept, 'immunostimulatory virotherapy', using rSeVs, which are recombinant virus-based immune boosters for DCs.[14,15] DCs activated by an rSeV have shown apparently superior antitumor effects on several tumor types compared with those seen by DCs treated with conventional stimuli, including lipopolysaccharide; they have also been shown not to lose their phago/pinocytotic activity,[15] and therefore an i.t. injection of rSeV-DCs without exposure to tumor antigen *ex vivo* evoked tumor-specific antitumor immunity.[14,15] On the basis of these findings, the present experimental study was performed to examine the potential of DC-based immunotherapy boosted by a newly developed rSeV/dF, the less cytotoxic, clinically available vector ts-rSeV/dF,[18] to treat less-immunogenic murine c1300 neuroblastoma.

The key observations obtained in this study were as follows: (1) c1300 tumors were highly resistant to ts-rSeV/dF-DC therapy by the 'early' treatment regimen that was sufficiently effective against highly malignant B16F10 melanoma, indicating that c1300 should be less immunogenic than such melanoma; (2) the use of ts-rSeV/dF-mIFNβ as the activating modality for DCs and expressing murine IFN-β dramatically attenuated the antitumor effect on c1300 tumors through the 'early' treatment regimen, similar to a finding of our earlier study;[14] (3) when established c1300 tumors were treated through the 'later' treatment regimen, however, the antitumor effect of ts-rSeV/dF-mIFNβ-DCs was not sufficient; (4) radiation pretreatment at a clinically reasonable dose (4 Gy × 3 days) demonstrated a dramatically improved antitumor effect, resulting in a high percentage of elimination of established tumors; and (5) the elimination of established c1300 tumors by radiation followed by ts-rSeV/dF-mIFNβ-DCs contributed to the development of long-lasting tumor-specific immunity, whereas the antitumor effect through overdose irradiation did not. These results indicate the potential utility of the combination of radiotherapy and ts-rSeV/dF-mIFNβ-DC immunogene therapy in the clinical setting.

It has been suggested that radiotherapy for malignancies might stimulate antitumor immunity as a systemic bystander effect called the 'abscopal effect'. However, the molecular and cellular mechanisms underlying this effect are largely unknown. In addition, the concept of combining radiation therapy with immunotherapy is not new, and some publications suggest the beneficial effect of irradiation on antitumor immunity. Radiation induces cell death through apoptosis and necrosis. In turn, necrotic and apoptotic cells could induce DC-mediated antitumor immunity.[29–31] These types of cell death are also shown not only to induce the release of inflammatory cytokines,[32] but also to stimulate tumor vasculature to upregulate the expression of adhesion molecules, and to facilitate the trafficking of immune cells to cancer foci; thus, these types of cell

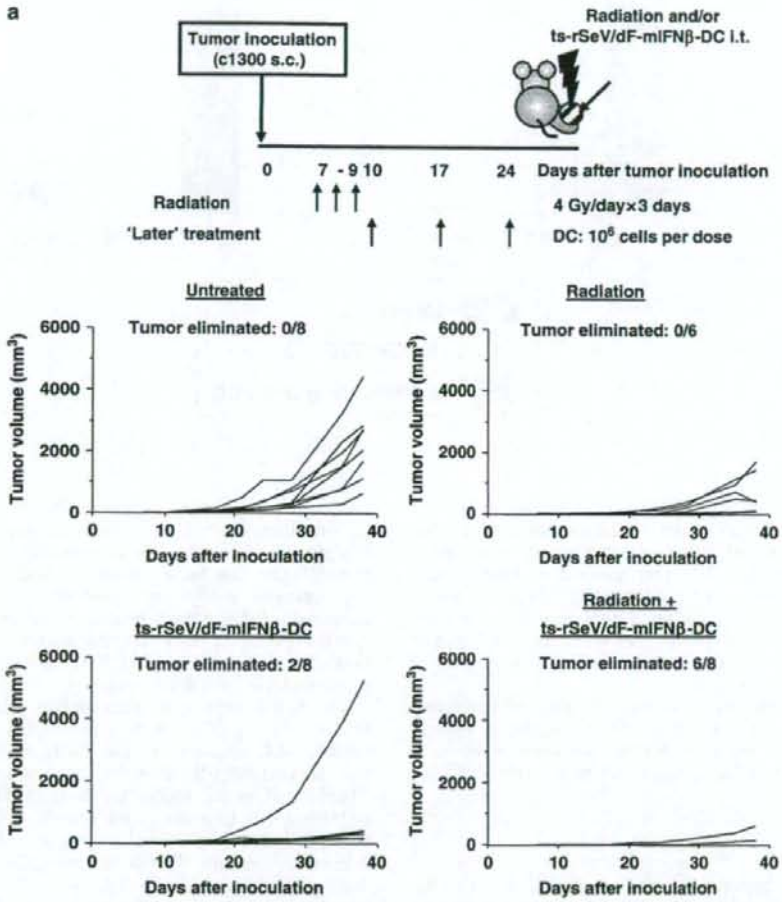


Figure 4 Pretreatment of clinically available dose of irradiation dramatically enhanced antitumor effect of ts-rSeV/dF-mIFNβ-DCs seen in the 'later' treatment regimen. Seven days after s.c. tumor inoculation into the left thigh, the tumors were irradiated three times daily at 4 Gy day^{-1}. At day 10 ('later' treatment regimen), 10^6 cells of ts-rSeV/dF-DCs with exogenous mIFN-β expression were injected weekly through the i.t. route. Thereafter, the tumor volume was measured. Note that all tumors treated in the 'later' regimen were over 7 mm in diameter at day 10. (**a**) Time courses of tumor volume in individual animals. No tumor disappeared in the group receiving radiation only, but tumor growth was suppressed (right upper panel) compared with those without any treatment (left upper). In contrast, the ts-rSeV/dF-mIFNβ-DC group showed a strong suppression of tumor growth (representative findings seen in Figure 3), and this effect was dramatically enhanced by the combination therapy, which resulted in the elimination of a high percentage of tumors (6/8 = 75%). (**b**) Direct comparison of tumor size. Panels showing c1300 tumor volume on day 38 in the animals demonstrated in (**a**). Note that animals showing a complete elimination of tumors were excluded in these analyses. Treatment with either irradiation or ts-rSeV/dF-mIFNβ-DCs significantly inhibited tumor growth, and synergism was found by the combination therapy. *$P<0.001$ and *$P<0.05$. (**c**) Long-term survival of animals demonstrated in (**a**). Two animals in the rSeV/dF-mIFNβ-DC group that showed the complete disappearance of tumors survived over 200 days (2/8 = 25%), whereas no significant prolongation of survival was seen in the group treated with irradiation alone. The combination therapy dramatically and significantly improved the survival of animals; six of eight animals (75%) survived over 200 days without any recurrence. The data were analyzed by the Kaplan–Meier method, and statistical relevance was determined using the log-rank test. *$P<0.001$. DC, dendritic cell; i.t., intratumoral; mIFN-β, murine interferon-β; rSeV, recombinant Sendai virus; s.c., subcutaneous injection. (See online version for color figure.)

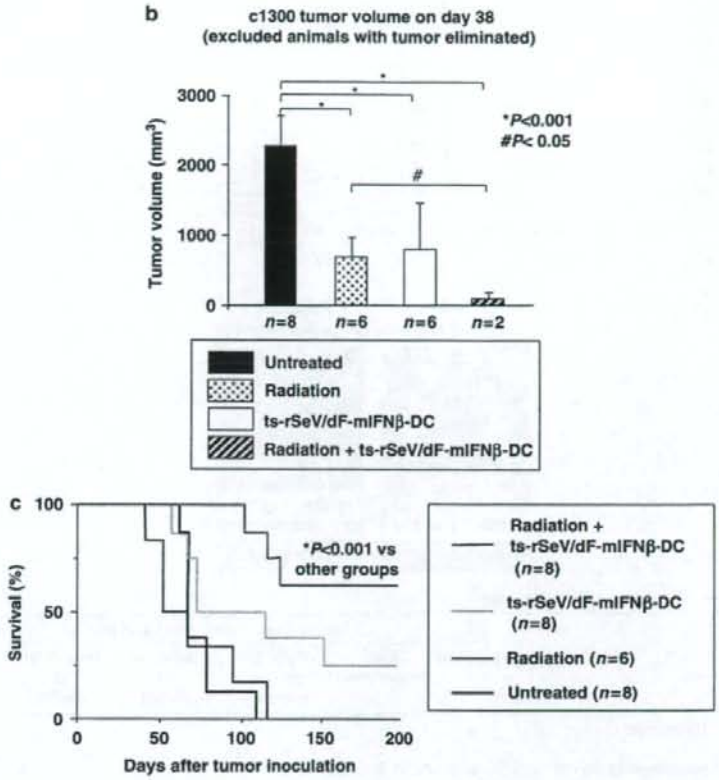


Figure 4 Continued.

death may together be responsible for the abscopal effect after radiation therapy.

It is of interest, however, that abscopal regression of distant tumors has been inferred in the use of certain tumors in experimental studies, but has rarely been seen in clinical settings.[33] In fact, we here demonstrated that tumor elimination through overdose irradiation did not protect against the second challenge of c1300 inoculation; rather, the addition of DC immunotherapy was required to establish a protective immunity. These findings could be supported by an important report describing that the induction of the T-lymphocyte-mediated abscopal antitumor response was tumor type specific.[25] Therefore, we concluded that DC-based immunotherapy would be required to induce protective immunity when tumor cells were destroyed by irradiation.

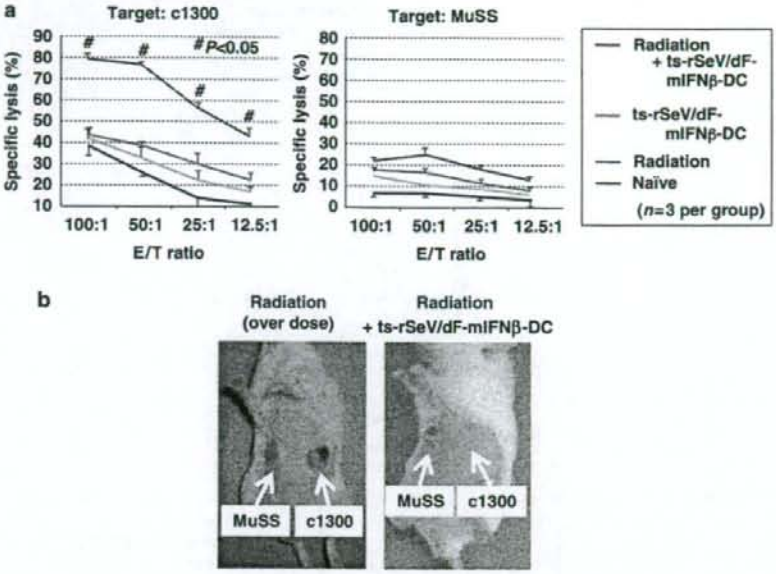
Related to this point, we have to discuss why c1300 tumor elimination through overdose irradiation (34 Gy × 3) did not induce protective immunity against the second challenge. In this case, a sustained dermal inflammation and burns were found (data not shown), possibly implying that the sustained dermal inflammation due to overdose irradiation might disturb the establishment of antitumor immunity. Therefore, clinically appropriate doses of irradiation, probably associated with tumor cell disruption proper for antigen uptake, processing and presentation by antigen-presenting cells, should be examined to obtain optimized antitumor immune responses following additional DC immunotherapy.

In summary, we here demonstrated that the i.t. injection of rSeV/DCs expressing mIFN-β to established c1300 tumors pretreated with a clinically reasonable dose of irradiation efficiently led tumors to a complete elimination *in vivo*. In addition, this regimen simultaneously induced a long-lasting protective immunity. Therefore, this results strongly suggest that the regimen warrants further investigation in research as well as in clinical trials.

Materials and methods

Mice and tumor cell lines

Female 6- to 8-week-old A/J mice (H-2^a) were obtained from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan) and kept under specific pathogen-free and humane conditions. Murine neuroblastoma c1300 and MuSS murine malignant fibrous histiocytoma (under kind permission by Dr Itaru Watanabe, Department of Surgery, Tasuda Hospital)[26] were obtained from the RIKEN BioResearch Center (Tsukuba, Ibaraki, Japan). B16F10 murine melanoma, SK-N-SH human neuroblastoma without *MYCN* amplification and IMR32 human neuroblastoma



Summary of second challenge

	Total examined	Second challenge (c1300 and MuSS)			
		Alive (tumor free)	Used for second challenge	Rejection of c1300	Rejection of MuSS
Untreated	4	0	-	-	-
Radiation (4 Gy×3)	3	0	-	-	-
Radiation (over dose: 34 Gy×3)	4	3	3	0	0
ts-rSeV/dF-mIFNβ-DC	4	1	1	0	0
Radiation (4 Gy×3) + ts-rSeV/dF- mIFNβ- DC	4	3	3	3	0

Figure 5 Combination therapy of irradiation and rSeV/dF-mIFNβ-DC induces long-lasting and tumor-specific protective immunity. (**a**) Assessment of CTL activity for c1300. Induction of tumor-specific CTLs after i.t. administration of rSeV/dF-mIFNβ-DC, which was repeated twice according to the late treatment regimen. Control included tumor-bearing mice without any treatment and MuSS was also used as a target of a third party. Seven days after the last treatment, splenocytes were isolated and restimulated *in vitro* for 5 days with mitomycin C-treated MH134 cells, and cytolytic activity against ^{51}Cr-labeled targets was measured. Each group contains $n=3$. (**b**) Seven days after s.c. c1300 tumor inoculation into the left thigh, the tumors were irradiated three times daily at 4 Gy day^{-1}. At day 10 ('later' treatment regimen), 10^6 cells of ts-rSeV/dF-DCs with exogenous mIFN-β expression were injected weekly through the i.t. route. Four animals with completely eliminated tumors through overdose irradiation (34 Gy × 3 for 3 days) were also included. On day 184, live animals without primary tumor formation were subjected to a second challenge, simultaneous tumor inoculation with c1300 and MuSS (third party) on the abdominal wall. Fifteen days later, tumor formation was determined. DC, dendritic cell, i.t., intratumoral; mIFN-β, murine interferon-β; rSeV, recombinant Sendai virus; s.c., subcutaneous injection. (See online version for color figure.)

associated with *MYCN* amplification were purchased from ATCC (Manassas, VA, USA). These cell lines were maintained in complete medium (RPMI-1640 medium; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum (BioWest, Nuaille, France), penicillin and streptomycin under a humidified atmosphere containing 5% CO_2 at 37 °C.

Temperature-sensitive mutant non-transmissible rSeVs (ts-rSeV/dF)

Temperature-sensitive mutant F-defective non-transmissible recombinant rSeVs (ts-rSeV/dF-null and ts-rSeV/dF-mIFNβ) were prepared and recovered as described earlier.[21,22] Briefly, vectors were prepared by using recombinant LLC-MK_2 cells carrying the F gene (LLC-MK_2/F7). An adenovirus vector, AxCANCre, expressing Cre recombinase was used for the induction of F protein in LLC-MK_2/F7 cells (referred to as LLC-MK_2/F7/A). Recombinant vaccinia virus vTF7-3 carrying T7 RNA polymerase was inactivated with psoralen and long-wave ultraviolet irradiation and then used for the ribonucleoprotein complex recovery. The viral vectors were further amplified by several rounds of propagation. The titers of the recovered viral vectors were expressed as cell infectious units.[9] Murine IFN-β cDNA, which was

subcloned to the vector template ts-prSeV18+b(+)/dF, was cloned by reverse transcriptase PCR as described earlier.[14]

Generation of DCs and transfection with rSeVs

Murine bone-marrow-derived DCs were generated as described earlier,[14,15] and an endotoxin-free condition was maintained throughout the study by using endotoxin-free reagents. Briefly, bone marrow cells from A/J mice were collected and passed through a nylon mesh, and red blood cells and lineage-positive (B220, CD5, CD11b, Gr-1, TER119, 7/4) cells were depleted by using the SpinSep mouse hematopoietic progenitor enrichment kit (StemCell Technologies, Vancouver, British Columbia, Canada). These lineage-negative cells ($5–10 \times 10^4$ per 5 ml per well) were cultured in 50 ng ml^{-1} granulocyte-macrophage colony-stimulating factor (PeproTech, London, UK) and 25 ng ml^{-1} IL-4 (PeproTech) in endotoxin-free complete medium in six-well plates. On day 4, half of the culture medium was replaced by fresh medium supplemented with granulocyte-macrophage colony-stimulating factor and IL-4 at the same concentration. On day 7, DCs were collected and used for subsequent experiments. For ts-rSeV-mediated transduction, DCs (1×10^6 cells per ml) were simply incubated with rSeVs at a dose optimized earlier[14,15] and a multiplicity of infection of 100 (MOI = 100) without any supplementation. At this condition, gene transduction efficiency constantly showed over 95%. Note that the DCs used in this study were not loaded tumor antigens, as was also the case earlier.[15]

Major histocompatibility complex class I expression on tumor cells

c1300 (mouse), SK-N-SH (human) or IMR32 (human) neuroblastoma cells (1×10^5 per ml) were incubated in the presence or absence of mouse or human IFN-β (1000 U ml^{-1}) at 37 °C for 48 h. These cells were then stained with the corresponding fluorescein isothiocyanate-conjugated anti-major histocompatibility complex class I antibodies (mouse or human; BD Pharmingen, San Diego, CA, USA) and were analyzed using FACS Calibur (Becton Dickinson, San Jose, CA, USA) with CellQuest software (BD Biosciences Japan, Tokyo, Japan). Data analysis was performed using FlowJo 4.5 software (Tree Star, San Carlos, CA, USA). Dead cells were excluded by staining with propidium iodide.

DC-based immunotherapy to c1300 tumor

The DCs used in this study were not pulsed with any tumor antigen throughout the experiments.

'Early' treatment regimen. After the DCs were prepared, an immature DC phenotype appeared constantly. These immature DCs were incubated with ts-rSeV/dF-null or ts-rSeV/dF-mIFNβ for 8 h, as described earlier.[14,15] All of the DCs were added to 50 mg ml^{-1} of polymyxin B (Sigma-Aldrich) and were carefully washed twice before injection. Intradermal implantation (A/J for 1×10^6 of c1300 cells showing log-phase proliferation *in vitro*) was performed into the abdomen on day 0, and 1×10^6 DCs were injected i.t. on days 3, 10 and 17. For all injections, materials were suspended in a 100-μl volume of phosphate-buffered saline. Tumor size was assessed using microcalipers three times a week, and the volume was calculated by the following formula: tumor volume (mm^3) = 0.5236 × (long axis) × (short axis) × (height) (Figures 1 and 3).[14,15]

'Later' treatment regimen and radiation pretreatment. We further assessed the 'later treatment regimen' for tumors that were well established, measured 7–9 mm in diameter[14,15] and constantly showed a significant vascularization histologically (data not shown). γ-Irradiation pretreatment (^{60}Co source, 2 Gy day^{-1} for 3 days, daily) was performed if necessary.

Dendritic cells were collected as described above, and intradermal implantation (A/J for 5×10^5 c1300 cells) was carried out into the abdomen (Figure 1) or right thigh (Figures 3–5: to avoid irradiation-induced enterocolitis and so on) on day 0, and 1×10^6 DCs were injected i.t. on days 10, 17 and 24. Tumor size was assessed as described above.

^{51}Cr release assay for cytolytic activity of CTLs

Prepared DCs were i.t. administered twice into tumor-bearing mice (MH134) at 10^6 cells per 100 μl on days 10 and 17. One week after the last immunization, splenocytes were obtained and contaminated erythrocytes were depleted. For CTL assay, 4×10^6 splenocytes were cultured with 3×10^5 inactivated c1300 cells treated with 100 μg ml^{-1} mitomycin in a 24-well culture plate. Two days later, 30 IU ml^{-1} human rIL-2 was added to the medium. After 5 days, the cultured cells were collected and used as CTL effector cells. Target cells (c1300 cells or MuSS for third party) were labeled with 100 μCi $Na_2^{51}CrO_4$ for 1.5 h, and Cr release assay was performed as described earlier.[14,15] The percentage of specific ^{51}Cr release of triplicates was calculated as follows: ((experimental c.p.m. × spontaneous c.p.m.)/(maximum c.p.m. × spontaneous c.p.m.)) × 100. Spontaneous release was always <10% of maximal Cr release (target cells in 1% Triton X-100).

Rechallenge of tumor cells

Nineteen animals bearing c1300 tumors were divided into five groups (Figure 6) (untreated: $n = 4$; 4 Gy × 3 days radiation: $n = 3$; 34 Gy × 3 days overdose radiation: $n = 4$; ts-rSeV/dF-mIFNβ-DC: $n = 4$; and 4 Gy × 3 days radiation+ts-rSeV/dF-mIFNβ-DC: $n = 4$) and treated as described in the schematic regimen in Figure 4. Seven animals had survived tumor-free on day 189 (34 Gy × 3 days overdose radiation: $n = 3$; ts-rSeV/dF-mIFNβ-DC: $n = 1$ and 4 Gy × 3 days radiation+ts-rSeV/dF-mIFNβ-DC: $n = 3$), and these were used for the second challenge. On day 189, 5×10^5 cells of c1300 (left) and MuSS (right) were inoculated into the bilateral dermis of the abdominal wall. Fifteen days later, tumor formation was assessed.

Statistical analysis

All data were expressed as means ± s.e.m. and were analyzed by one-way analysis of variance with Fisher's adjustment, except for animal survival. Survival was plotted using Kaplan–Meier curves, and statistical relevance was determined using log-rank

comparison. A probability value of $P<0.05$ was considered significant.

Abbreviations

ts-rSeV, temperature-sensitive mutant recombinant Sendai virus; ts-rSeV-DC, recombinant Sendai virus-modified DC; IFN-β, interferon-β; i.t., intratumoral injection; s.c., subcutaneous injection'

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Competing interest

Dr Yonemitsu is a member of the Scientific Advisory Board of DNAVEC Corporation.

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■ 特集 小児がん治療の晩期障害と対策

進行神経芽腫の TBI 併用大量化学療法

七野浩之*

I. 小児がん長期生存者

小児がんと診断されて治療を受け成人した「小児がん経験者」は，小児がん治療の進歩につれますます増加している。これらの人々は欧米では childhood cancer survivors とか long term survivors のように survivors と呼ばれる。日本では小児がん「経験者」,「克服者」,「長期生存者」などのようにまだ一定の表現に落ち着いていない。本稿では，小児がんに打ち克ち成人になり長期生存を続けているという点に注目して,「小児がん長期生存者」を使用する。

また,「晩期」という用語には，もう終わりに近いという感覚があるため関係者の間では議論になっている。いわゆる「晩期障害」には，重篤な身体的障害や二次がん発症による長期的な生命予後の不良という問題が，従来より重要な問題として認識されてきていた。近年ではそのほかにも精神的障害や社会的適応の不具合や周囲の人々との関係あるいは日常生活の不具合なども重要な問題として再認識されてきており，実にさまざまな問題が起こっていることがわかってきた。本稿では「晩期障害」に対しては，小児がん長期生存者にみられる「長期的な問題点」という用語を使用することとする[1]。

II. 長期的な問題点と Erice 宣言

小児がんの多くの疾患は，現在では 5 年生存率が 70%を超え，80〜90%に迫る疾患もみられるようになった[2]。その結果，アメリカ合衆国では成人期を迎えた小児がん長期生存者は 30 万人に上り，全成人の 350〜600 人に 1 人と推定されている[2]。日本もそれに近い長期生存者がいると予測される。

これまで多くの小児がん医療関係者は，まずは小児がん患者の生存を第一と考えてきた。また治療終了後も，再発の早期発見早期再治療を第一の課題と考え力を注いできた。そのため，小児がん長期生存者に生じている「長期的な問題点」に対する認識が十分ではなかったと思われる。

小児は，身体的精神的に成長発達の途上で発病し治療を受けるため，非常に影響が大きいことは想像に難くない。小児がん長期生存者に対して，病気から立ち直り，十分に機能を回復し，望ましい生活の質を確保し，自立した一人の成人として同年代の人々と同じように社会に受け入れられるように援助する体制[3]については，多くの医療関係者がその必要性を痛感しながらも，多くの労力を割くことができず，整備が遅れていた。漸くこの 2〜3 年になりその重要性必要性が急速に社会的に注目され，その体制整備の機運が盛り上がってきている。

国際ベルリン-フランクフルト-ミュンスター (I-BFM) グループは，2006 年 10 月に「小児がん長期生存者：治癒と支援 (long term survivors of childhood cancer：cure and care)」についての Erice 宣言[3]を発表し，小児がんを経験した長期生存者に対する長期にわたる治療後の健康面での経過観察と支援体制の必要性を全世界に向け強く訴えた。そのなかで，小児がんやその治療に関連して起こるあるいは起こりうる長期的な影響 (long-term effect) について，医療関係者は注目し，長期経過観察と支援のための専門外来 (follow-up clinic) で，種々の専門家をチームの一員

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とした援助体制を作る必要性を掲げている[3]。さらには，これらの長期的な影響については，小児がん患者本人と家族に十分に説明されなければならないことも強調されている[3]。

III. 進行神経芽腫の長期生存者

神経芽腫は，脳腫瘍を除く 0 歳から 15 歳までの小児固形腫瘍の 7〜10%を占め，アメリカ合衆国では年間約 800 人，日本では年間約 200 人と推定されている。そのうち進行神経芽腫は日本では 60 人程度と推定される。現在の日本の進行神経芽腫患者の生存率は明らかではないが，これまでの各施設の治療成績や厚生省班会議の治療成績などをもとに大まかに推測すると大体 30%程度と多くの研究者は考えている。この比率であれば進行神経芽腫の長期生存者は年間 20 人前後の計算となり，20 年間では単純に 400 人と推測される。

進行神経芽腫の患者の多くは，成長発達にとって重要な時期である 5 歳以下で発見される。この時期に家族や社会から隔絶された環境で長期間入院を強いられ，強力な化学療法や全身放射線照射(TBI) を含む放射線療法や外科療法が行われるため，短期的な問題はもちろん，さまざまな身体的精神的社会的な「長期的な問題点」が生じている。

現在までに進行神経芽腫に行われてきた世界的な標準的治療戦略は，多剤併用寛解導入化学療法→二期的外科切除術（→放射線療法）→多剤併用強化化学療法→地固め療法としての骨髄破壊的大量化学療法±TBI を用いた造血細胞救援療法という治療骨格である。化学療法には，シスプラチン，トポイソメラーゼII阻害剤，アントラサイクリン系薬剤，ビンクリスチン，シクロフォスファミドのうちから 3〜5 種類の薬剤を選択し組み合わせた多剤併用療法が 5〜7 回程度行われている。さらに全身的な微小転移巣とくに骨・骨髄転移の根絶を期待して，10〜12 Gy の分割照射による TBI を併用した骨髄破壊的大量化学療法による自家造血幹細胞救援療法が行われている。このような TBI を併用した大量化学療法レジメンを鍵とした治療戦略の有効性が示され生命予後はそれ以前よりも改善はしたが，一方で TBI による急性毒性に加えて，成長障害，内分泌障害，白内障，二次がんなどの「長期的な問題点」の発生頻度は決して低くなく，神経芽腫の長期生存者にとって大きな問題であることが明らかにされた[4]。

IV. 小児がん長期生存者全体としての長期的な問題点：CCSS からの研究成果

神経芽腫も小児がんのひとつである。まずは小児がん長期生存者全体にみられる長期的な問題点を解説する。日本にはまだまとまった研究報告がないため，1993 年から北米で開始された the Childhood Cancer Survivor Study（CCSS）からの研究成果をもとに問題点を明らかにする。これは 1970 年 1 月 1 日から 1986 年 12 月 31 日に診断された 21,655 人の小児がん経験者を対象にした前向きコホート研究としての長期追跡調査研究である[1,5]。

1. 長期生命予後

小児がん長期生存者の死亡率は同じ年齢の小児がん非経験者の対照群に比べ高いことが明らかにされた。小児がん診断後の生存率は緩やかに下がり，診断後 5 年以上生存していた小児がん長期生存者群では，30 年後では生存率は約 80%に低下している。同じ年齢の対照群では 95%以上である[1,6]。死亡原因として最も多いのは原疾患の再発(死亡原因の 58%) であり，おおむね診断 15 年後までは再発率は増加しているが，15 年以降は心障害（7.0%）や呼吸器障害（1.8%）などの長期的な問題点による死亡が増加し，さらには二次がんによる死亡率が 14.8%の高率に上っている[1]。

2. 二次がん

累積二次がんの発症率は，診断後 10 年では 2%であるが，20 年で 5%，30 年で 15%と非常に高率である[7]。腫瘍のうち骨腫瘍，乳癌，甲状腺癌，脳腫瘍，白血病などにとくに注意が必要である。発症までの年数（中央値）は，2 次がんとしての白血病，脳腫瘍，軟部腫瘍，黒色腫などは原発がん診断後 10 年以内にみられ，骨腫瘍，乳癌，甲状腺癌などは診断後 10 年以上である。放射線照射歴のある群での二次がん累積発症率はさらに上昇し，30 年後には 20%に近い累積発症率である。とくに甲状腺癌は放射線照射歴により危険性が高くなる。神経芽腫は，甲状腺癌，褐色細胞腫，脳

腫瘍，急性白血病，骨髄異形成症候群，骨肉腫，乳癌そして，腎癌の危険性が高い[1,4]。

3．身体的な長期的な問題点

診断後 25～30 年で約 77%の小児がん経験者がなんらかの Common Terminology Criteria for Adverse Events (CTCAE) で評価される身体的有害事象を発症していた[1,8]。しかもその 38%が CTCAEver3.0 でグレード 3 または 4 に分類されるような重度の有害事象であった。重度な身体的有害事象は，当然放射線治療や抗癌剤治療を受けた群で高くなっている。また，これらの治療も，単独治療よりも併用療法のほうがリスクが高い。アントラサイクリン系薬剤＋アルキル化剤，胸部＋腹部放射線照射，腹部照射＋アルキル化剤，胸部照射＋アントラサイクリン系薬剤の併用で危険度が高い[1]。

経時的な身体的有害事象の発生頻度は，診断から 5 年以内では 70%以上の長期生存者でほとんど有害事象を認めないが，診断後 5 年以降に急速に増加しはじめ，30 年後にはすべてのグレードの有害事象で 70%，グレード 3～4 の高度な有害事象で 30%に上る[1]。

4．経過観察中断の問題点

より幼少なときに診断される脳腫瘍や神経芽腫では本人が正確な診断名を知らない比率が高い。このことは長期経過観察が中断される要因のひとつである。実際小児がん診断後 10 年で 50%の，20 年で 80%の患者は小児がんに関係した受診をしなくなっていた。また，近隣のプライマリーケア医へ受診する率は 60%と比較的保たれているが，プライマリーケア医では小児がんに関連した相談を受けることが難しいことが明らかにされている[1]。

V．TBI 併用大量化学療法による進行神経芽腫の長期生存者の長期的な問題点

これまでの報告では次のような長期的な問題点が指摘されている。甲状腺機能低下症，成長ホルモン分泌不全，糖尿病，思春期発来障害などの内分泌障害。肥満や痩せの成長障害。心筋障害，不整脈などの心機能の障害。腎機能障害。聴力障害。白内障などの眼領域の障害。側彎症，下肢長の不均等，骨粗鬆症，筋力の低下，骨塩の低下などの筋骨格系障害。歯牙の成長発達障害。神経精神系の障害。ボディーイメージの障害，などである[4,5]。

これらの問題点の頻度としては，例えばメモリアル スローン ケタリングがんセンターからの報告[9]では，聴力障害 62%，甲状腺機能低下症 24%，卵巣機能不全 41%，筋骨格系障害 19%，肺障害 19%が認められた。多くは中等度の障害であり，生命の危険性があるような重篤なものは 4%であった。シスプラチンを使用された患者では聴力障害が多かった。シクロフォスファミド使用量が 7.4 g 以上では卵巣機能不全を伴ったと報告されている。

また，フランスからの報告[10]では，TBI の影響は身長と体重の成長障害に最も影響を強く及ぼした。その他の障害は，甲状腺，白内障，2 次がんである。聴力障害と歯牙形成不全もみられると報告された。

1．成長障害

成長障害としては低身長が多く認められる。主として TBI による成長ホルモン (GH) 分泌障害と考えられているが，非 TBI 群でも程度は軽いが認められている。GH 以外の甲状腺機能低下症や性腺機能低下症あるいは，栄養障害や骨成長障害など種々の原因が考えられる。TBI 群では，移植後 5 年後の身長は－2.0 SD，非 TBI 群では 10 年後－0.7～－0.9 SD との比較もある[11]。

GH の分泌不全が関与していれば，GH 補充療法が効果を発揮しており，最終身長を伸ばすためには，可能な限り早期から，GH 投与量を最大にすることが推奨されている[5]。

男性では栄養障害からのやせが問題となる一方，肥満が問題となることも同様に指摘されている[5]。低身長と体重増加が組み合わされると，体重自体は標準より小さくても脂肪肝などの肥満による合併症が認められる例もあり，問題は複雑である。

神経芽腫長期生存者は，甲状腺機能低下症や性腺機能低下症などを含め，視床下部や下垂体のホルモン分泌不全を認めることが多い。これらのホルモンについての基礎値の測定や負荷試験が必要であり，その結果を受けて，ホルモン補充療法を

積極的に行う必要がある。

2．学習障害や生活の質の障害

プラチナ系抗がん剤による聴力障害は，高音域から障害が始まり，次第に低音域へと下がってくることが特徴である。英語圏では，s，f，th などのすれる音や破裂する音には高音域の成分が多く含まれるため，会話領域の聴力障害となる。このことから，読む，計算する，そのほかの一般的な学習障害や特別な学習に障害が引き起こされることが明らかとなった。アンケート調査による自己評価でも，両親の評価でも，学習や精神的社会的な問題が多くみられ，生活の質の低下につながる深刻な問題となっている[12]。日本ではまとまった報告がないが，長期経過観察のなかで会話領域の聴力障害を訴える長期生存者は多い。

3．歯の成長発達障害

これまであまり問題視されなかったが，重要な問題として，進行神経芽腫で大量化学療法を受けた患者は，歯の成長発達障害を認めることが明らかとなった。歯根の短縮や，歯根発達の停止，歯牙の縮小，歯の再生不良である[13]。TBI 群では 10 人中 9 人で，2～12（平均で 6.6）本の永久歯の欠失を認めるが，非 TBI 群では 5 人中 2 人に 2 本と 4 本の永久歯の欠失を認めたのみであった。

VI. 日本大学における TBI を併用した大量化学療法後の進行神経芽腫患者の長期的な問題点の検討

日本大学医学部附属板橋病院では 1999～2005 年に 15 人の進行神経芽腫患者を治療し，12 人に大量化学療法を施行した。大量化学療法後現在生存している 8 人のうち，2 年以上経過している 6 人を対象として長期的な問題点について検討した。男 3 人，女 3 人，移植時年齢は 1～8 歳，中央値は 6 歳である。病期は 3 期が 1 人，4 期が 5 人である。観察期間は 2 年 8 カ月～6 年 9 カ月である。大量化学療法はメルファラン＋エトポシド＋カルボプラチン併用療法を行い，3 期の 1 人以外の 5 人に 10 Gy の全身放射線照射を施行した。

その結果，6 人とも現時点で神経芽腫の再発はない。大量化学療法後，4 人に慢性上顎洞炎を認め繰り返している。低身長を 4 人に認め，成長ホルモン補充療法を 3 人に行っている。甲状腺機能低下症を 5 人に認め，3 人に甲状腺剤の補充療法を行っている。1 人の女性に性腺機能低下症を認め補充療法を行っている。2000 Hz 以上の高音域を主とした感音性難聴を 6 人全員に認めている。白内障を 3 人に認めている。血液検査および尿検査で異常を認める腎機能障害を 3 人に認めている。6 人に発毛障害を認め，うち 1 人は重症である。現在までのところ不整脈や心機能低下はみられていない。免疫，内分泌，耳鼻，眼，腎，心等のなんらかの長期的な問題点が全員に認められている。

おわりに

これまでみてきたように，小児がん長期生存者にみられる長期的な問題点は種々に及んでいる。とくに TBI を含む大量化学療法による長期的な問題点は大きな問題として認識されてきている。そのため欧米諸国ではすでに 10 年近く前から非 TBI による治療戦略を採用しその結果も以前と比較して遜色のない結果を報告している。日本でも現在日本神経芽腫研究グループにより非 TBI による臨床研究が進行中である。われわれは，急いで，また継続して，日本の小児がん長期生存者の長期的な問題点を明らかにし，その治療や援助に当たるとともに，より長期的な問題点の少ない新たな治療戦略の開発を進めることを迫られている。

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Long-term Problems in Long-term Survivors of Advanced Neuroblastoma Treated with High-dose Chemotherapy with Total Body Irradiation

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Key words : Long-term problems, Children, Advanced neuroblastoma, High-dose chemotherapy, Total body irradiation.
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Intensive chemotherapy, high-dose chemotherapy with or without total body irradiation, surgery, and local radiotherapy are providing long-term survival in many children with advanced neuroblastoma. Long-term survivors are at high risk for many long-term problems, including early death, second cancer, pshychosocial disturbance, and organ system dysfunction, and especially for endocrine, musculoskeletal, neurologic, sensory, cardiac, and pulmonary impairments. We summarize these long-term problems in this analysis.

* * *

Circulating Methylated-*DCR2* Gene in Serum as an Indicator of Prognosis and Therapeutic Efficacy in Patients with *MYCN* Nonamplified Neuroblastoma

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Abstract **Background:** *MYCN* amplification (MNA) in neuroblastoma is a strong indicator of poor prognosis. However, some *MYCN* nonamplified (non-MNA) cases show poor outcomes, and examining the status of the gene requires an operation, which may have surgical complications. Therefore, a new marker is needed to identify cases of non-MNA neuroblastomas with poor prognoses using less risky procedures. Aberrant hypermethylation of the *DCR2* promoter has recently been associated with rapidly progressing neuroblastoma. We aimed to develop a noninvasive *DCR2* methylation assay for patients with neuroblastoma using serum DNA, which predominantly originates from tumor-released DNA.

Methods: Using DNA-based real-time PCR, we simultaneously quantified a methylated-*DCR2* specific sequence (M) and a reference sequence (R) located in the promoter region in serum DNA, and evaluated *DCR2* methylation status as M/R ratios in 86 patients with neuroblastoma.

Results: Serum *DCR2* M/R ratios were strongly correlated with those in the tumor ($r = 0.67$; $P = 0.002$). *DCR2* methylation was associated with stage both in the whole neuroblastoma group and in the non-MNA group ($P < 0.001$), and *DCR2*-methylated patients showed significantly poorer 5-year event-free survival in the whole neuroblastoma group (43% versus 84%; $P < 0.001$), especially in the non-MNA group (12% versus 96%; $P < 0.001$). Among five *DCR2*-methylated patients whose clinical courses were followed, serum M/R ratios were close to 0 in the patients in remission, whereas the ratios increased in patients who relapsed.

Conclusions: Detection of methylated-*DCR2* in serum DNA has promise as a noninvasive assay for predicting prognosis and therapeutic efficacy in neuroblastoma, especially in non-MNA cases. Furthermore, it might be a sensitive marker of tumor recurrence in *DCR2*-methylated cases.

Neuroblastoma is the most common extracranial solid tumor in children and is characterized by a wide range of clinical behaviors, from spontaneous regression to rapid progression with a fatal outcome (1). The current risk classification of patients with neuroblastoma is based on the age of onset, the extent of disease at the time of diagnosis as defined by the International Neuroblastoma Staging System, and evaluation of genetic aberrations, such as *MYCN* amplification (MNA; ref. 2). MNA is considered the strongest prognostic factor and is routinely assessed for therapy stratification (2–4), but it is a big concern that some cases without MNA also have a poor prognosis (5). Hero and colleagues recently reported that spontaneous regression is often seen in localized infantile neuroblastoma without MNA, and they suggested that a wait-and-see strategy avoiding chemotherapy and surgical procedure was justified in such patients (6). Nevertheless, some patients in their cohort showed local progression, progression to stage IVS, or progression to stage IV. Thus, it is important to have additional biomarkers with prognostic value for the management of non-MNA cases of neuroblastoma. We screened many biological markers for neuroblastoma, such as Trk A expression (7) and chromosome allelic loss (8–10), but none of them were found to be useful for risk classification among non-MNA patients.

Recent studies have revealed that epigenetic alterations, such as silencing of tumor suppressor gene by aberrant hypermethylation of its promoter, often play important roles in the pathogenesis of human cancers, and some of these alterations are thought to cause loss of function comparable with

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